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Biofilm dispersal for spore release in *Bacillus subtilis*

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ABSTRACT

The dispersal of bacterial cells from a matured biofilm can be mediated either by active or passive mechanisms. In this issue of the *Journal of Bacteriology*, Nishikawa and Kobayashi demonstrate that the presence of calcium influences dispersal of spores from the pellicle biofilm of *Bacillus subtilis*. The authors propose that temporal heterogeneity in matrix production and chelation of calcium by dipicolinic acid in spores weakens the biofilm matrix and causes passive dispersal.

KEYWORDS

Bacillus subtilis, biofilm, dispersal, spore, development

COMMENTARY

Biofilm formation is a complex developmental process undertaken by microbes that is initiated by attachment or aggregation of cells, advanced by production of an extracellular matrix, and

generally finalized by disassembly of the biofilm, a process called dispersal (1). The specific biofilm life cycle depends on the microorganism, its ecological niche, and the encoded regulatory pathways. In addition, environmental factors, including intra- and interspecies compounds may influence the different steps of biofilm development. A detailed understanding of the different stages of biofilm formation and disassembly could help us to prevent deleterious microbial communities and promote beneficial ones.

Bacillus subtilis became a model organism to study bacterial differentiation processes due to its ability to create a dormant cell structure, called a spore, that has remarkably resistance to heat, pressure, and chemicals in addition to its capability to take up extracellular DNA and incorporate it via recombination into its genome (2). These features, alongside other biotechnologically beneficial properties stimulated robust probing of the physiology and genetics of this species in the last century. The study of *B. subtilis* biofilm formation was initiated about two decades ago, and has created a plethora of understanding since the first publication (3) regarding how gene expression connects to biofilm initiation and matrix production and the identity and function of the main biofilm matrix components (4, 5). Interest in *B. subtilis* biofilms is further stimulated by the species being more than a laboratory model: biofilms are important for plant growth promotion, probiotic impact, and biotechnological applications (6, 7).

The molecular details of *B. subtilis* biofilm development have been predominantly explored in two laboratory systems, air-liquid interface floating biofilms, known as pellicles, and architecturally complex colonies formed on agar surface (6). Dissection of gene expression in colonies revealed that *B. subtilis* biofilm population is phenotypically heterogeneous; distinct cell types inhabit a biofilm, including motile cells, matrix producers, extracellular protease producers, and in the later stages of development, spores are formed on the upper layer of the colonies (8, 9). Efficient

47 initiation of pellicle development requires motility (10) and establishment of the floating biofilm at
48 the air-medium interface proceeds through distinct morphological changes (11). Importantly,
49 matrix gene expression in the nascent pellicle is temporally and spatially heterogeneous, after a
50 highly heterogeneous matrix production during initiation of the pellicle, the majority of cells
51 express the genes for matrix production in the middle of biofilm development (around 24 hours)
52 (12). In the later stages, the population becomes heterogeneous again, only a fraction of the cells
53 will produce the matrix (12), while spores also appear (13, 14). Such temporal heterogeneity is
54 mirrored by physical heterogeneity; during the initial and later stages of pellicle development,
55 next to a robust, highly matrix-expressing population, a fragile fraction is also present, within
56 which the cell-cell aggregation can be easily disrupted (12). The dynamic transcriptional landscape
57 of the developing pellicle has also been associated with variation in metabolism of the cells (15).
58 Additionally, it has been proposed that during biofilm colony maturation, the evolutionary
59 younger and more diverged genes are increasingly expressed toward later timepoints of colony
60 development (16).
61 While initiation and maturation of pellicle biofilm development is extensively investigated,
62 dispersal mechanisms are less explored in *B. subtilis* and the literature still perpetuates errors in
63 the understanding of biofilm dispersal with respect to norspermidine and D-amino acids that have
64 since been corrected (17, 18). In this issue of *Journal of Bacteriology*, the publication by Nishikawa
65 and Kobayashi (19) reveals a novel mechanism of *B. subtilis* dispersal and highlights a connection
66 between emergence of spores and biofilm disassembly (Fig. 1). Interestingly, *B. subtilis* grown in
67 variety of commonly used synthetic and complex biofilm media (e.g. MSgg and 2×SGG) does not
68 display typical dispersal. The pellicle remains robust for up to a week at 30°C, during which time
69 only a minor and very slow decay in the thickness is observed in MSgg grown pellicles. However,

70 pellicles that were cultivated at 37°C in a modified LBGM medium (lysogeny broth supplemented
71 with glycerol and manganese (20), but containing reduced amount of manganese compared to
72 previous publications) showed a rapid establishment within a day, fragmented structure on the
73 second day, and strong dispersal after 3 days. While removal of manganese prevents biofilm
74 development (20, 21), supplementation at lower concentration creates conditions that allows
75 examination of the full pellicle biofilm life cycle in *B. subtilis*, including dispersal. These
76 observations highlight that biofilm dispersal might be more prevalent in the laboratory when slight
77 starvation is encountered, a condition that likely exists for microbes in nature. Interestingly, the
78 number of viable cells remained constant throughout the 3 days (19), suggesting the lack of active
79 lysis or cell death, but rather the presence of passive dispersal in *B. subtilis* succeeding the
80 previously reported reduced matrix gene expression at later stages of the pellicle development
81 (12, 15). Synthetic induction of genes involved in synthesis of the exopolysaccharides throughout
82 the cultivation and therefore prolonged exopolysaccharide production partially prevents biofilm
83 dispersal (19), thus the reduced matrix production only partially explains pellicle biofilm dispersal.
84 What could facilitate *B. subtilis* cells' dispersal from a biofilm in addition to reduced matrix
85 expression? *B. subtilis*, when colonizing plant roots under hydroponic conditions, first produces
86 the biofilm matrix followed by robust spore formation (22). Spore are anticipated to survive the
87 harsh conditions in the soil, including predation by protozoans, nematodes, or other microbes
88 (23–25). Therefore, pellicle dispersal could understandably be mediated by release of spores.
89 Nishikawa and Kobayashi (19) demonstrate that the induction of sporulation pathway, which
90 depends on a cascade of sigma factors activating specific gene expression either in the mother cell
91 (σ^E, and σ^K) or in the pre-spore (σ^F, σ^G), contributes to dispersal. Indeed, circumventing spore
92 formation by disrupting these sporulation-specific sigma factors, in addition to concomitantly

93 synthetically prolonging exopolysaccharide production, prevents pellicle dispersion. Systematic
94 disruption of σ^K-dependent genes, the last downstream sigma factor within the activation
95 cascade, revealed that *spoVFA–spoVFB* operon is sufficient to explain biofilm dispersal in *B.*
96 *subtilis*. The *spoVFA–spoVFB* operon encodes a dipicolinic acid synthase that creates dipicolinic
97 acid (DPA). DPA after being produced in the mother cells is transported to the forespore
98 compartment where it starts chelating calcium ions contributing to dehydration and
99 mineralization of the spore (26). The direct connection between DPA mediated chelation of
100 calcium, and pellicle dispersal could be verified by addition of calcium to the biofilm medium,
101 which prevented dispersal (19). The impact of calcium on biofilm colony development has been
102 previously observed (27). Consistently, both Nishikawa and Kobayashi (19) and Mhatre *et al.* (27)
103 could demonstrate that calcium does not impact the expression of matrix genes in established
104 pellicle biofilms and under biofilm inducing conditions, respectively, besides both studies reported
105 larger biofilm colony size in the absence of calcium. The larger colony size observed in the absence
106 of calcium is likely connected to passive surface spreading, termed sliding, as the influence of
107 calcium on colony size was only apparent in the presence of matrix components and surfactin that
108 are all necessary for sliding (27–29). Production of the secondary metabolite surfactin, while not
109 being essential for biofilm development, alters the architecture of biofilm colonies (30). Calcium
110 influences self-assembly of surfactin (31), which might explain a possible impact of calcium on
111 surface tension and therefore biofilm colony size (27). Nevertheless, it remains to resolve the
112 direct connection between calcium level and surfactin functioning in colonies.
113 Thus, calcium has an important role both in spore maturation and also influences dispersal.
114 Nishikawa and Kobayashi (19) offer a plausible explanation how spore formation indirectly
115 influences biofilm weakening. Besides lower production of the matrix, the onset of spore

maturation and accompanying DPA production depletes the calcium in the extracellular matrix, resulting in biofilm dispersal (Fig. 1). Calcium seems to impact the biofilm matrix and/or influence regulation of biofilm in numerous bacteria (32–34). It remains to demonstrate whether and how calcium directly interacts with the biofilm matrix in *B. subtilis*. Nevertheless, the elegant work by Nishikawa and Kobayashi describes an intriguing example of passive dispersal and connects spore formation with it release from the biofilms.

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Figure 1

Schematic representation of the *B. subtilis* pellicle biofilm life-cycle. After inoculation of planktonic cells, oxygen depletion drives the motile cells to the air-medium interface, where biofilm formation is initiated. At the start, part of the population produces the biofilm matrix. During biofilm maturation, most cells expend energy making the biofilm matrix and calcium is distributed across the biofilm, possibly stabilizing the matrix structure. Before dispersal, matrix production diminishes, and spores are formed that chelate available calcium. Calcium depletion and reduced matrix production allow passive dispersal of *B. subtilis* pellicle biofilms. Figure created with BioRender.com